

Attenuation of Nuclear Factor Kappa B (NF- κ B) Promotes Apoptosis of Kidney Epithelial Cells: A Potential Mechanism of Mercury-Induced Nephrotoxicity

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Nuclear factor kappa B (NF- κ B), a pleiotropic transcriptional factor that promotes cell survival and protects cells from apoptosis, requires reduced thiols at critical steps in its activation pathway. Mercuric ion (Hg^{2+}), one of the strongest thiol-binding agents known, impairs NF- κ B activation and transcriptional activity in normal rat kidney epithelial (NRK52E) cells at concentrations as low as 0.5 μM by binding to specific reduced thiol moieties in the NF- κ B activation pathway. We hypothesized that prevention of NF- κ B activation by Hg^{2+} will increase the sensitivity of kidney cells to the apoptosis-inducing effects of other toxicants to which these cells are otherwise resistant by virtue of their NF- κ B-activating capacity. Fewer than 5% of untreated kidney cells in culture (70–90% confluent) were found to be apoptotic when evaluated by DNA fragmentation (terminal deoxynucleotide transferase-mediated dUTP nick-end labeling) or flow cytometric DNA profile analyses. Hg^{2+} (5 μM) treatment for 24 hr increased this proportion by 1.5- to 2-fold. Neither lipopolysaccharide (LPS) (1 $\mu\text{g}/\text{mL}$) nor tumor necrosis factor- α (TNF- α ; 300 U/mL), both potent activators of NF- κ B in kidney cells, significantly altered the proportion of apoptotic cells, compared with untreated controls, when applied without Hg^{2+} pretreatment. However, when LPS or TNF- α was administered after Hg^{2+} pretreatment (5 μM for 30 min), the proportion of cells undergoing apoptosis 22 hr later increased by 4- to 6-fold compared with untreated controls. In contrast, Hg^{2+} pretreatment did not increase the amount of apoptosis caused by apoptosis-inducing agents that do not activate NF- κ B (staurosporine, Fas ligand). These findings suggest that Hg^{2+} enhances the sensitivity of kidney cells to apoptotic stimuli as a consequence of inhibition of NF- κ B activity. Because apoptosis is known to play a key role in the pathogenesis of renal failure resulting from toxicant injury to proximal tubular cells, promotion of apoptosis via inhibition of NF- κ B activity may define a novel molecular mechanism by which Hg^{2+} toxicity is initiated in kidney cells. **Key words:** apoptosis, kidney, mercuric ion, mercury, nephrotoxicity, nuclear factor- κ B. *Environ Health Perspect* 110(suppl 5):819–822 (2002).

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Nuclear factor kappa B (NF- κ B) is a pleiotropic transcriptional factor involved in the inducible expression of a wide variety of genes, particularly those that promote cell growth and survival and that protect cells from apoptotic death stimuli (1). NF- κ B is a heterodimer most frequently comprising a 50-kDa protein called p50 or NF- κ B1 and a 65-kDa protein referred to as p65 or *relA*. In most mammalian cells the NF- κ B heterodimer resides in the cytoplasm in the inactive form, bound to an inhibitor protein, I κ B. Upon exposure of the cell to a variety of stimuli such as growth factors, bacterial lipopolysaccharide (LPS, endotoxin), or cytokines such as tumor necrosis factor- α (TNF- α), inhibitory kappa B (I κ B) is phosphorylated and ubiquitinated, allowing it to be degraded by the 26S proteasome. This results in release of the active NF- κ B heterodimer, which then translocates to the nucleus and mediates the upregulation of specific pro-survival and anti-apoptotic genes by binding to the κ B consensus sequence in their regulatory regions.

Many of the steps in the signaling pathway leading to NF- κ B activation are known to possess conserved cysteine moieties and to

require reduced thiol groups for optimal signal transduction and DNA binding (2). In previous studies (3,4), we have demonstrated that NF- κ B is readily activated by LPS and TNF- α in normal rat kidney epithelial cells. Moreover, we have shown that mercuric ion (Hg^{2+}), one of the most potent thiol-directed agents known, attenuates NF- κ B activation in kidney cells at concentrations as low as 2 μM (i.e., ~ 2 nmol/mg protein). These effects of Hg^{2+} are directed toward impairment of I κ B phosphorylation, translocation of p65 to the nucleus, and NF- κ B–DNA binding. These actions result in attenuated NF- κ B transcriptional activity, as determined in kidney cells transiently transfected with an NF- κ B–driven luciferase reporter plasmid (pLuc-4 \times NF- κ B) (4,5).

In the studies described here, we investigated the implications of Hg^{2+} -mediated attenuation of NF- κ B activity in kidney cells with respect to Hg^{2+} -induced nephrotoxicity. Underlying this investigation are two basic tenets. The first is that apoptosis plays an important role in the pathogenesis of those forms of renal failure in which tubular epithelial cells are the primary target of toxicant

injury (6), as occurs with Hg^{2+} exposure (7). The second is that NF- κ B prevents apoptosis in numerous cell types by promoting the upregulation of specific anti-apoptotic gene products, such as Bcl-2 and the zinc finger protein A20 (8,9). Because Hg^{2+} impairs NF- κ B activation at cellular concentrations that do not prevent cellular energy production (i.e., < 20 μM), we hypothesized that Hg^{2+} promotes cell death by increasing the sensitivity of kidney cells to the apoptosis-inducing effects of agents such as TNF- α and LPS, to which kidney cells are normally resistant because of their ability to otherwise activate NF- κ B.

Materials and Methods

Materials

Lipopolysaccharide from *Escherichia coli* (serotype 026:B6) and HgCl_2 (Sigma M1136) were acquired from the Sigma Chemical Co. (St. Louis, MO, USA). TNF- α (human recombinant, *E. coli*, catalog no. 654205; 1 ng = 110 WHO IU) was obtained from Calbiochem (La Jolla, CA, USA). All other chemicals were purchased from standard commercial sources and were of the highest available purity.

Cell Culture and Treatments

NRK52E cells, a clonal line established from normal rat tubular epithelial cells (ATCC no. CRL-1571), were acquired from the American Type Culture Collection (Manassas, VA, USA) and were propagated in Dulbecco's modified Eagle's medium with high glucose, pyruvate, and L-glutamate (Cambrex; Biowhittaker, North Brunswick, NJ, USA) supplemented with 5% heat-inactivated

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newborn calf serum (GIBCO; Invitrogen, Carlsbad, CA, USA) plus 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma). Cell monolayer mats were grown to confluence in 6- and 12-well plates (Costar, Corning, NY, USA) and also in 25- and 75-cm² cant-necked, vent-capped, uncoated flasks (Costar). Medium was replaced every 2–5 days, irrespective of state of confluency. Treatments were performed when cells covered 70–90% of flask surface.

Electrophoretic Mobility Shift Assays and Autoradiography

Electrophoretic mobility shift assays (EMSAs) to demonstrate activation of NF-κB in nuclear protein extracts were performed as previously described (3,4).

Apoptosis Assays

Apoptosis of NRK52E cells was detected by the terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) method, using a fluorescein-based TUNEL cell death detection kit (Boehringer Mannheim, Indianapolis, IN, USA). The percentage of apoptotic cells was calculated using a RT slider spot camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) and NIH Image software (Universal Imaging Corp., Downingtown, PA, USA). Additional apoptosis assessments were performed by flow cytometric analysis of cellular DNA content after fixed cell preparations were stained with 4,6-diamidino-2-phenylindole (DAPI) (10). Analyses were performed on an ELITE cytometer (Coulter Corp., Miami, FL, USA) using ultraviolet laser excitation. Acceptable histograms were derived from a minimum of 10,000 cells and contained a coefficient of variation below 6%. DNA content histograms and cell cycles were analyzed using the MultiCycle software program (Phoenix Flow Systems, San Diego, CA, USA). The percentage of apoptotic cells was quantified as the percentage of total cells displaying reduced DNA staining, the sub-G₀/G₁ peak. Before analysis, cellular debris was gated out on the basis of cell scatter.

Statistical Analyses

Analysis of differences between treatment groups for EMSA assays was determined using a paired, one-tailed *t*-test. The level of significance was chosen at <0.05.

Results

Hg²⁺ Attenuates NF-κB Activation in Kidney Epithelial Cells

In previous studies (3) we have demonstrated activation of NF-κB by various agents (e.g., LPS, TNF-α, thapsigargin) in kidney epithelial cells and have shown that this effect is

defined by specific activation of the p50p65 heterodimer. More recently we have demonstrated that activation of NF-κB by such agents is readily attenuated in a dose-related manner after pretreatment of cells with low Hg²⁺ concentrations (4). Thus, as shown in Figures 1 and 2, LPS-induced and TNF-α-induced activation of NF-κB was significantly impaired after pretreatment of cells with Hg²⁺ at concentrations as low as 0.5 µM. In additional studies, we have observed similar effects of Hg²⁺ on NF-κB activation by LPS or TNF-α in adult human renal tubular epithelial cells (Clonetics RPTC 5899, Cambrex), as well as by TNF-α in primary rat astrocytes (data not shown). These findings attest to the non-cell-specific and non-species-specific nature of this effect.

Hg²⁺ Induces Apoptosis in Kidney Epithelial Cells

Within the concentration range over which Hg²⁺ attenuates NF-κB activity (<20 µM), Hg²⁺ elicits a small but discernable increase in apoptosis in kidney cells. As shown in Figure 3, the proportion of cells undergoing apoptosis increased from fewer than 5% in untreated cultures (Figure 3A) to 8–10% when evaluated by the TUNEL assay 22 hr after 5 µM Hg²⁺ treatment (Figure 3B). Evidence of Hg²⁺-induced apoptosis was also obtained when evaluated using flow cytometric techniques in which the proportion of cells that fell within the sub-G₀/G₁ fraction of the total cell population was identified as apoptotic. Representative plots of diploid cell cycle analysis in which DNA content from untreated (Figure 3C) and Hg²⁺-treated (Figure 3D) cell cultures was measured after DAPI staining are also shown in Figure 3. We observed similar apoptotic effects after treatment with Hg²⁺ at 10 µM; however, no increased apoptosis was found after treatment with Hg²⁺ at 2 µM (not shown).

Hg²⁺ Pretreatment Enhances the Sensitivity of Kidney Cells to Apoptotic Stimuli That Also Activate NF-κB

Although a small increase in the proportion of apoptotic cells was found after treatment with Hg²⁺ alone, pretreatment of kidney cells with Hg²⁺ at concentrations that impair NF-κB activation significantly increased the sensitivity of cells to the apoptosis-inducing effects of other agents to which these cells are otherwise resistant because of their capacity to activate NF-κB. Thus, as shown in Figure 4A, TNF-α (300 U/mL) elicited no increase in the proportion of apoptotic cells compared with untreated controls, when evaluated by the TUNEL assay 22 hr after treatment. In contrast, when TNF-α was administered to cells 30–45 min after treatment with Hg²⁺

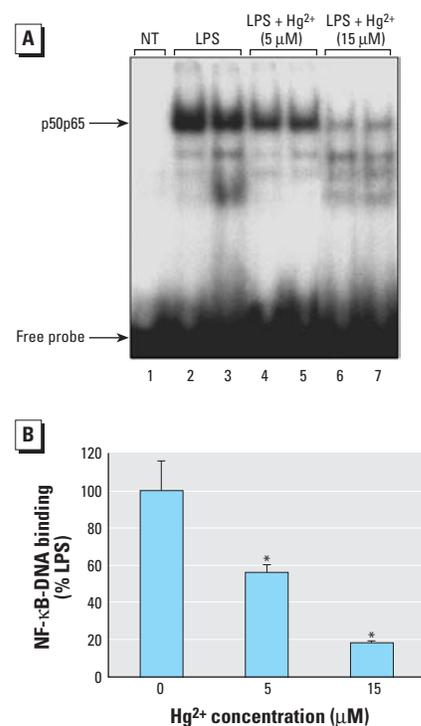


Figure 1. Effects of Hg²⁺ on LPS activation of NF-κB in NRK52E cells. (A) EMSA showing dose-response effects of Hg²⁺ on p50p65-DNA binding when given to cells 30 min before LPS (1 µg/mL) administration. NT, not treated. (B) Binding signals are presented as a percentage of that for LPS alone. Error bars show standard deviation. **p* < 0.05.

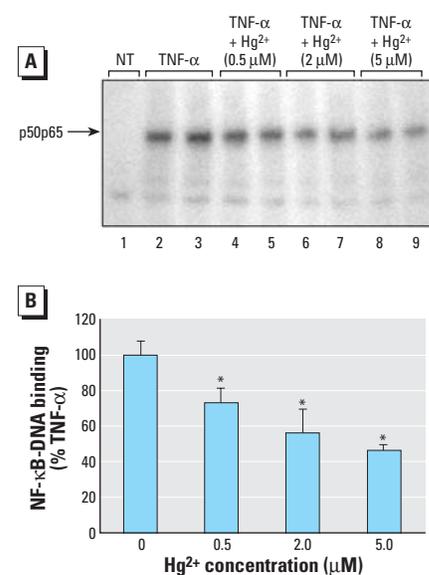


Figure 2. Effects of Hg²⁺ on TNF-α activation of NF-κB in NRK52E cells. (A) EMSA showing dose-response effects of Hg²⁺ on p50p65-DNA binding when given to cells 30 min before TNF-α (300 U/mL) administration. (B) Binding signals are presented as a percentage of that for TNF-α alone. Error bars show standard deviation. **p* < 0.05.

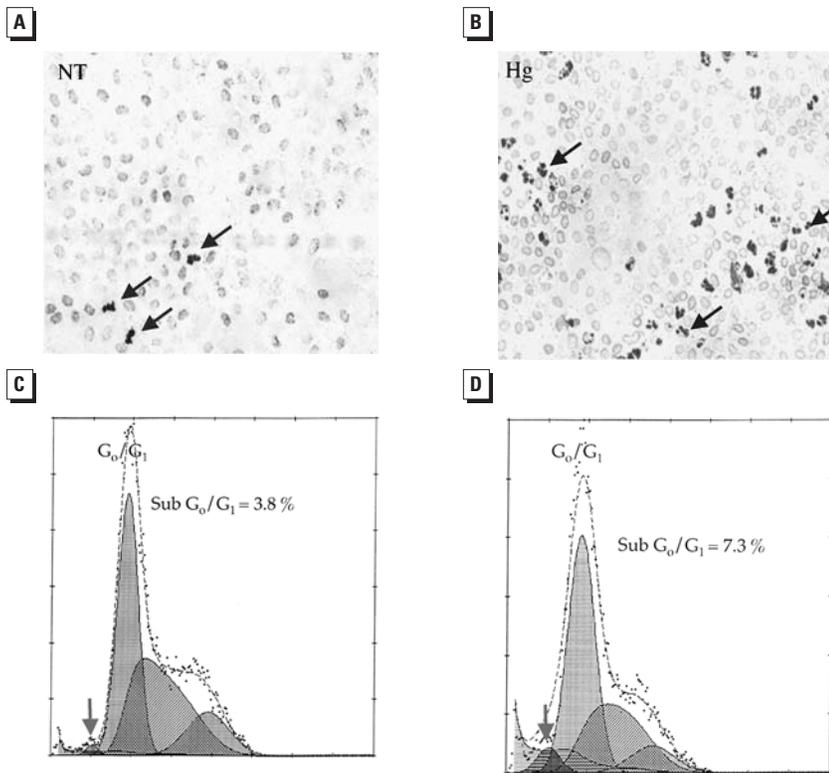


Figure 3. Apoptosis in NRK52E cells after Hg²⁺ treatment. Cells were untreated or treated with 5 μ M Hg²⁺ 22 hr before harvesting. Apoptosis was evaluated using the TUNEL assay (A and B) or by flow cytometry (C and D), as described in "Materials and Methods." Arrows in A and B represent TUNEL-positive cells. Arrows in C and D represent the sub-G₀/G₁ cell fraction.

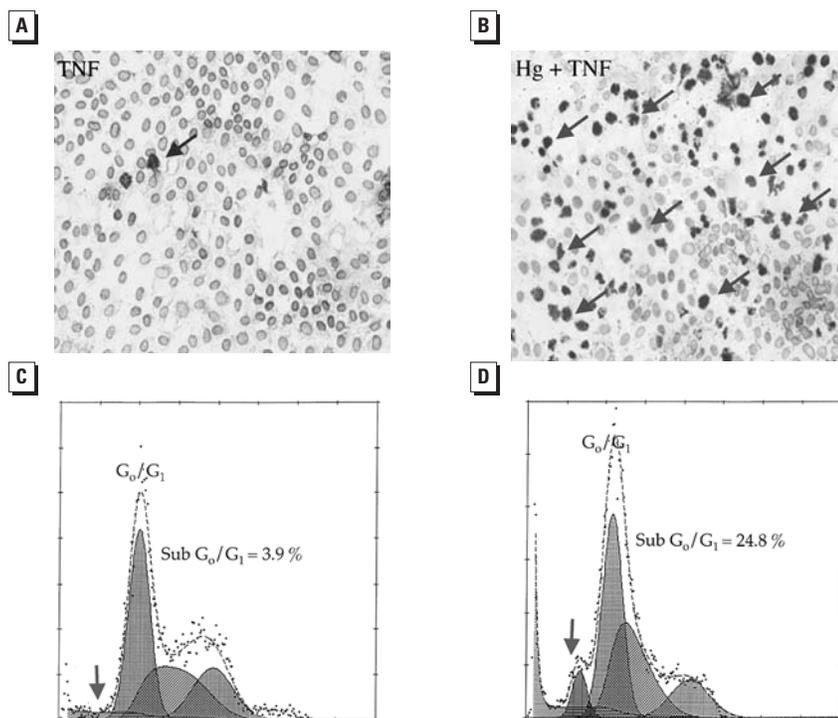


Figure 4. Enhancement of TNF- α -induced apoptosis in NRK52E cells by Hg²⁺. Cells were treated with 5 μ M Hg²⁺ 30 min before TNF- α (300 U/mL) or with TNF- α alone. Cells were harvested 22 hr after TNF- α administration. Apoptosis was evaluated using the TUNEL assay (A and B) or by flow cytometry (C and D), as described in Materials and Methods. Arrows in A and B represent TUNEL-positive cells. Arrows in C and D represent the sub-G₀/G₁ cell fraction.

(5 μ M) (Figure 4B), a dramatic increase (>6-fold) in apoptosis was observed. Similar findings were obtained when apoptosis was evaluated using flow cytometry, as described for Figure 3. The proportion of apoptotic cells increased from fewer than 4% after treatment with TNF- α alone (same proportion as seen in untreated cells) to 25% when TNF- α was given after Hg²⁺ administration (Figure 4C, D). Notably, Hg²⁺ pretreatment increased the sensitivity of cells to the apoptotic effects of TNF- α even when administered at concentrations (i.e., <5 μ M) that were not sufficient to elicit a significant increase in apoptosis by Hg²⁺ alone (not shown), supporting the view that the effects of Hg²⁺ and TNF- α in this effect are interactive rather than independent mechanistic events.

The augmentation of sensitivity to the apoptosis-inducing effect of NF- κ B-activating agents by Hg²⁺ was not restricted to TNF- α . We obtained similar findings when we substituted LPS for TNF- α in comparable experiments (Table 1). Notably, apoptosis has been implicated as an important mechanism of cell death *in vivo* after LPS exposure (11). As shown in Table 1, treatment with LPS (1 μ g/mL) alone increased the proportion of apoptotic cells to approximately 8% when measured 22 hr after administration. When LPS was applied 30 min after Hg²⁺ pretreatment (5 μ M), the amount of apoptosis increased to more than 35% of total cells.

In contrast Hg²⁺ pretreatment did not substantially increase the amount of apoptosis found after treatment of cells with apoptosis-inducing agents that do not concomitantly activate NF- κ B. Staurosporine (200 nM), which initiates apoptosis by inducing the mitochondrial permeability transition (12) without activating NF- κ B, elicited a significant increase in the proportion of apoptotic cells (27.9%), and pretreatment with 5 μ M Hg²⁺ did not significantly increase the effect of subsequently administered staurosporine (Table 1). We obtained similar findings with anti-Fas ligand

Table 1. Hg²⁺ augments apoptosis of kidney cells by agents that activate NF- κ B.^a

Agent	% Apoptotic cells	
	No Hg ²⁺ pretreatment	Hg ²⁺ pretreatment
None (control)	3.8	7.3*
TNF- α	3.9	24.8*
LPS	8.2	35.1*
Staurosporine	27.9	29.3
Anti-Fas ligand	67.8	68.5

^aApoptosis was determined in NRK52E cells by the TUNEL assay as described in "Materials and Methods." Hg²⁺ (5 μ M) pretreatment was performed 30 min before administration of the indicated agent. TNF- α (300 U/mL), LPS (1 μ g/mL), staurosporine (200 nM), or anti-Fas ligand (1 μ g/mL) was administered 22–24 hr before apoptosis determinations. *Significantly greater ($p < 0.05$) than no Hg²⁺ pretreatment ($p < 0.05$).

(1 µg/mL), which induces apoptosis by direct caspase activation after receptor engagement (13), without NF-κB activation.

Discussion

Hg²⁺ is a potent nephrotoxicant, with predominant effects directed toward proximal tubular epithelial cells of the S3 segment (pars recta) (7,14). Previous studies from this laboratory and others (15–18) have focused largely on disruption of cellular membranes and impairment of mitochondrial bioenergetics as principal events underlying Hg²⁺ toxicity. In contrast, few studies have sought to define the specific molecular mechanisms through which Hg²⁺ initiates toxicity via alteration of thiol-dependent signal transduction pathways that regulate cellular proliferation and survival. The present findings indicate that Hg²⁺, at cellular concentrations that do not compromise cellular energy production, impairs activation of NF-κB and that this effect may increase the sensitivity of kidney cells to the apoptosis-inducing effects of other toxicants and infectious agents to which kidney cells are otherwise resistant. Because apoptosis is thought to play an important role in the pathogenesis of renal failure associated with toxicant injury to tubular epithelial cells (6,9), the present findings suggest a novel molecular mechanism by which Hg²⁺ may initiate nephrotoxicity. Moreover, because NF-κB plays a key role in preventing apoptosis in numerous cell types, findings obtained in the present kidney cell model might be pertinent to the etiology of mercury toxicity in the central nervous system or other tissues in which mercury is preferentially accumulated.

Of considerable relevance to the present observations is the current increased concern regarding risks and susceptibility to toxicity that may result from population exposures to biological agents. The findings that low-level mercury exposure may increase the sensitivity of kidney cells to LPS-mediated toxicity raises the possibility that such exposure may enhance susceptibility to *E. coli* or other biological

agents in other organ systems as well. Notably, low-level mercury exposure has been reported to significantly enhance susceptibility to infection from encephalomyocarditis virus (19) and leishmaniasis (20) in mice and from pseudorabies virus in rabbits (21). These observations suggest that low-level mercury exposure may promote the toxic effects of biological agents at tissue concentrations that do not elicit overt Hg²⁺ toxicity per se. Confirmation of this hypothesis through further research would be of substantial public health significance because it would suggest potential health risks associated with considerably lower levels of Hg²⁺ exposure than are currently thought to be of public health concern.

In conclusion, the present studies provide evidence of a novel molecular mechanism through which Hg²⁺, by preventing NF-κB activation, promotes toxicity by increasing the sensitivity of kidney and possibly other tissue cells to the apoptosis-inducing effects of other agents. Further studies directed toward confirmation of this observation could provide a mechanistic basis for improved understanding of a wide range of human health disorders that are associated with mercury exposure.

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